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Sex ratio variation and sex determination in *Urtica dioica*

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c h a p t e r I

General introduction

'Dioecious plants offer in many cases better tools for studying the genetics of sex determination than dioecious animals. First, the fact that dioecious plants have arisen independently gives an opportunity to study the different ways in which dioecism may become established. Second, in plants the step from dioecism is not clear-cut. In many dioecious species [...] bisexual types are found in nature often with a rather high frequency. Such bisexual individuals of normally dioecious plant species are almost always fertile and can be studied genetically whereas similar bisexual animals are sterile intersexes. Finally, in plants it is possible to follow both the evolution of dioecism from hermaphroditism (or monoecism) and the reverse process.'

Westergaard 1958

The great majority of flowering plants produce hermaphrodite (perfect) flowers, which means that they develop flowers with both functional male and female organs. This situation contrasts strikingly with that in the animal kingdom. Here, most species are unisexual, and male and female gametes are produced by different individuals. In plants, only approximately 10% of the angiosperm species produces unisexual flowers (Yampolsky and Yampolsky 1922). Species with unisexual flowers can be divided into two main categories: monoecious and dioecious. With monoecy (e.g. *Zea mays*), male and female organs are carried on separate flowers on the same individual, whereas with dioecy (e.g. *Cannabis sativa*) male and female flowers are carried on separate male and female individuals. Beside those three sex systems, a number of other so-called polygamous sex systems exist which may be intermediates during the evolution of full unisexuality or may be stable forms. These are, among others, gynodioecy (female and cosexual [hermaphrodite or monoecious] plants; e.g. *Plantago coronopus*), androdioecy (male and cosexual plants; e.g. *Datista glomerata*), and sub-dioecy (male, female and cosexual plants; e.g. *Thalictrum*

spp.). The various modes of sex systems in flowering plants are shown in Figure 1.1.

Only about 6% of the angiosperm species are dioecious (Renner and Ricklefs 1995), yet dioecy is taxonomically distributed among most of the major orders of monocot and dicot angiosperms. Families with the highest concentrations of dioecious genera are the Menispermaceae (100% of the genera are dioecious), Myristicaceae (78%), Moraceae (62%), Urticaceae (52%), Anacardiaceae (50%), Monomiaceae (47%), Euporbiaceae (39%), and Cucurbitaceae (32%) (Renner and Ricklefs 1995). Ecological and morphological traits that were found to be significantly and strongly associated with dioecy are wind or water pollination, perennial growth and woodiness, inconspicuous flowers, fleshy fruits, climbing growth form, and tropical distribution (Renner and Ricklefs 1995). Some tropical floras are specifically rich in dioecious species, such as in Hawaii (27.8%) or in New Zealand (12-13%) (Bawa 1980). Examples of dioecious species are mistletoe (*Viscum album*), poplar (*Populus* spp.), willow (*Salix* spp.), white campion (*Silene latifolia*), annual mercury (*Mercurialis annua*), sorrel (*Rumex* spp.), stinging nettle (*Urtica dioica*), kiwifruit (*Actinidia deliciosa*), hop (*Humulus lupulus*), date palm (*Phoenix dactylifera*), and papaya (*Carica papaya*). Some of them are agronomically important, and often one sex is preferred.

Strictly dioecious plant species consist of entirely male and female individuals. In some dioecious species however, dioecy is not clear-cut and occasionally bisexual plants occur beside pure male and female individuals. This condition, termed sub-dioecy, applies for a number of plant species that are generally regarded as dioecious in the literature. For example, asparagus (*Asparagus officinalis*) can be considered to be a sub-dioecious species according to the definition: while females are consistent with their sexual phenotype, male plants range from fully male to hermaphrodite with substantial fruit production (Machon et al. 1995). Because such bisexual individuals are mostly fertile, they have been used to study the inheritance of sex in those species (reviewed by Westergaard 1958). Likewise, *U. dioica* can be regarded to be sub-dioecious because male plants have been observed that occasionally produce fruits (Kay and Stevens 1986, de Jong et al. 2005). However, in this thesis I use the term sub-dioecious only for *U. dioica* when we included male, female and bisexual individuals in our study.

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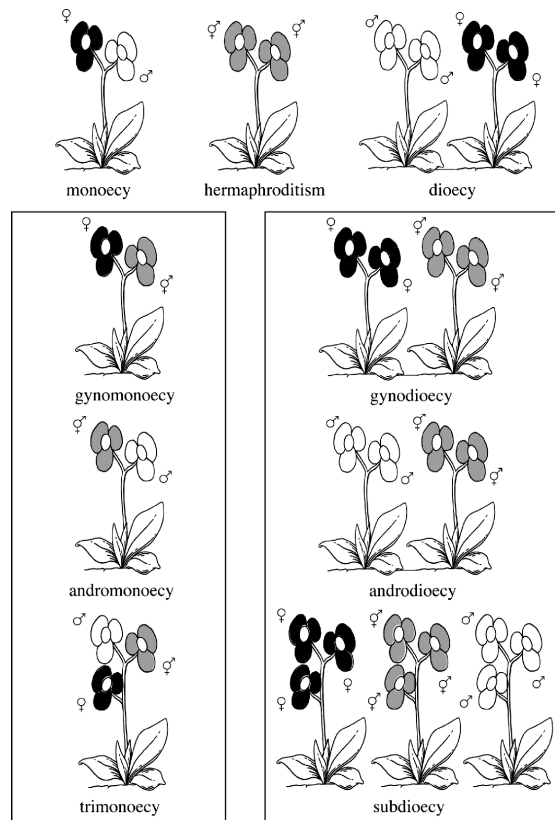


FIGURE 1.1 – Sexual systems in flowering plants. Various modes of mixed sex systems are shown in the boxes. *Gynodioecy*, *gynomonoecy* and *trimonoecy* are relatively common while *androdioecy*, *andromonoecy* and *sub-dioecy* (*trioecy*) on the other hand are rare breeding systems. Adapted from Fig. 1.1 with permission from de Jong and Klinkhamer (2005), *Evolutionary Ecology of Plant Reproductive Systems*, Cambridge University Press.

Monoecy: male and female organs are carried on separate flowers on the same plant

Hermaphroditism: plants with perfect (male and female reproductive organs) flowers

Dioecy: male and female flowers are carried on separate male and female individuals

Gynomonoecy: plants with both female and hermaphrodite flowers

Andromonoecy: plants with both male and hermaphrodite flowers

Trimonoecy: plants with female, male and hermaphrodite flowers

Gynodioecy: populations with female and hermaphrodite/monoecious plants

Androdioecy: populations with male and hermaphrodite/monoecious plants

Sub-dioecy (*Trioecy*): populations are composed of male, female and hermaphrodite/monoecious plants

SEX RATIOS

As compared to most animals, in dioecious plant species some offspring are male and others are female. Generally, the sex ratio is defined as the proportion of males to the total number of offspring. There are two distinct types of sex ratios, the individual sex ratio and the population sex ratio. The individual sex ratio which is also called primary sex ratio (in plants sometimes termed seed sex ratio or progeny sex ratio) refers to the sex ratio of the progeny that is produced by a female individual. In practice, the individual sex ratio is the sex ratio that is obtained after all seeds have been raised to mature plants with 100% germination and no mortality. In contrast, the population sex ratio (secondary sex ratio) refers to the sex ratio of adult individuals in the field that form a population. The secondary sex ratio can deviate from the initial sex ratio due to differences in mortality, longevity, timing of flowering, and vegetative reproduction between the two sexes (reviewed by Delph 1999). Also, the capability to change sex according to varying environmental conditions can alter the sex ratio of a population from season to season (Yamashita and Abe 2002). While in some dioecious plant populations the observed sex ratio bias may be a consequence of differential response of females and males to selective forces acting on certain life history traits, the sex ratio bias in populations of other species may be a result of a bias in the primary sex ratio.

For a long period of time it was assumed that in sexually reproducing species, males and females are produced in equal numbers giving a 1:1 sex ratio. Indeed for many species, this will be expected as an inevitable consequence of the chromosomal mechanism of sex determination. However, this view has changed after more and more studies revealed that the existence of sex chromosomes in a given species does not guarantee a 1:1 sex ratio. Species with sex chromosomes, both in plants and animals have evolved sex ratios that can differ considerably from 1:1. Although several authors made a similar argument earlier, Fisher (1930) is most frequently cited for providing a genetic explanation based on nuclear genes for the evolution of a stable sex ratio of 1:1, given that male and female offspring are equally costly to produce. Because every offspring has a maternal and a paternal parent, he assumed that both parents must contribute (on average) equally to subsequent generations and there-

fore must have the same average fitness in a panmictic (well-mixed) population. Fisher (1930) also argued that in populations with biased sex ratios, selection will favour the production of the minority sex, simply because individuals of the sex in demand have a higher reproductive success. However, since Fisher's (1930) assumptions only apply to panmictic, outcrossing populations and because many populations have been observed that persistently exhibit sex ratios deviating from 1:1, numerous mechanisms of selection favouring biased sex ratios have been proposed. These mechanisms include, for example, local mate competition (Hamilton 1967), maternal control (Trivers and Willard 1973), cytoplasmic elements (Uyenoyama and Feldman 1978), and sex-chromosome meiotic drive (Sandler and Novitski 1957). De Jong et al. (2002) considered a model, in which the population was not panmictic, but rather pollen and seed were allowed to disperse over certain distances. When mating occurs within local groups (sibs), selection favours autosomal (nuclear) genes to produce female biased sex ratios, as these reduce the competition for mates among genetically related males (local mate competition). On the other hand, the optimal sex ratio becomes balanced or even male-biased when pollen is dispersed over larger distances than seeds and the likelihood of non-local mating increases. Pollen dispersal reduces the possibility that related individuals need to compete for the same resources (local resource competition, de Jong et al. 2002). Furthermore, numerous authors showed experimentally in mammals that varying maternal conditions influence sex ratio among progeny as predicted by Trivers and Willard (1973). Although classic sex allocation theory (Charnov 1982) based on nuclear inheritance of autosomal genes and maternal control over sex ratio has been able to explain and to predict variation in primary sex ratios of animal species (see West et al. 2002), sex ratio data obtained so far from dioecious plant species are not in line with the theory (see de Jong and Klinkhamer 2005).

A more recent theory, taking the role of genetic conflict into account, may give more insight into the genetics of sex ratio variation (reviewed by Cosmides and Tooby 1981, and Werren and Beukeboom 1998). Genetic conflict occurs when different components of a genetic mechanism are subject to selection in opposite directions and may act between genomes (e.g. paternal-maternal conflict) or

within genomes (between genes located on autosomes and sex chromosomes or between cytoplasmic and nuclear genes). Genetic conflict in cosexual plants has received considerable attention in relation to gynodioecy, the occurrence of females (male sterile) and cosexuals within one population (reviewed by Samitou-Laprade et al. 1994). Male sterility is often caused by a cytoplasmic factor. Because cytoplasmic genes are mostly transmitted through seeds and not through pollen (Corriveau and Coleman 1989), these genes have higher transmission to subsequent generations and therefore selection will favour the existence of male sterile (=female) plants in the population. For example, if female plants produce 1.6 times as many seeds as hermaphrodites like in gynodioecious *P. lanceolata*, the cytoplasm causing male sterility transfers 60% more copies of itself than a neutral cytoplasm. The sex ratio bias in the population can be adjusted again, if specific nuclear genes that restore male fertility are present. In addition to work on natural plant populations, male sterility effects also received a lot of attention in crop species (Agrawal 1998). This is largely because of the usefulness of male sterility in plant breeding programs. For this reason, male sterility is often incorporated into one of the parental lines in the production of hybrid seed to ensure that none of the seed produced are the result of selfing.

The study of genetic conflict in relation to sex ratio in dioecious plant species is a relatively new approach. Potentially in dioecious species, genetic elements in male and female individuals may have conflicting interests with respect to reproductive outcomes (Cosmides and Tooby 1981). In *S. latifolia*, a dioecious plant species with morphological distinct sex chromosomes and male heterogamy, so called 'sex ratio males' were identified that produce little or no male progeny (Taylor 1994). Testing pollen fertility from male plants producing different sex ratios in their progeny, Taylor and Ingvarsson (2003) found a significant relationship between the fraction of fertile pollen and the progeny sex ratio. Particularly those 'sex ratio males' aborted almost half of their pollen. They interpreted the result in the way that the severe female-biased sex ratios produced by such males were due to abortion of Y-bearing pollen. This non-Mendelian process, which causes some alleles to be over-represented in the gametes that are formed during meiosis, is quite similar to sex-chromosome meiotic drive systems found in animals (e.g. Lyttle 1993,

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Silver 1993). Furthermore, conflict between nuclear genes and cytoplasmic genes is expected. Because cytoplasmic genes are most commonly transmitted through seeds and not through pollen (Corriveau and Coleman 1989), those genes transferred to male progeny are at a dead end. As a result, selection favours cytoplasmic genes to effectively bias the sex ratio towards femaleness (Taylor 1994).

MECHANISMS OF SEX DETERMINATION

The application of phylogenetic analyses left no doubt that dioecy has arisen independently in different plant families (Soltis et al. 1999) and plant genera (e.g. Weller et al. 1995, Desfeux et al. 1996). Given this fact the diversity of sex determination mechanisms in plants is not surprising. Many studies on sex determination of sexually dimorphic plant species have been made in the past century, enabling us to evaluate the factors influencing the sexual phenotype. It is now generally accepted that sex expression in plants can be determined solely or by an interaction of physiological, environmental and genetic factors (reviewed by Chailakhyan and Khrianin 1987, Meagher 1988, and Ainsworth 1998).

Physiological factors

There are two principle aspects of physiology of sex expression that have been studied in dioecious plant species: influences of plant growth substances (phyto-hormones) and differential enzyme activity. The first aspect will be discussed here. Analogous to animals, in which the importance of hormones in sex regulation has long been established, it was expected that phyto-hormones would play a significant role in plant sex regulation. In the course of investigation of the physiology of the sexual phenotype, the influence of almost every conceivable category of physiologically active substances on sex expression has been explored by exogenous application. In fact, such studies have been widespread and various plant hormones have been found that have a substantial influence on sex expression, both in dioecious and monoecious plant species (reviewed by Chailakhyan and Khrianin 1987). Basically, gibberellins have been shown to enhance male sex expression and studies on endogenous levels of gibberellins in male and female plants indeed provided evidence of the masculinisation effect (e.g. Leshem and Oplin 1977). Auxins, on the other hand,

have been demonstrated to enhance female sex expression in the majority of plants investigated. Furthermore, cytokinins have been found to play a role in sex expression (reviewed by Chailakhyan and Khrianin 1987). Their effects, however, are more variable than those of gibberellins and auxins. For example in *M. annua*, exogenous application of cytokinins provokes masculinisation of female plants. Femaleness in turn can be induced by auxins. Studies with dioecious *M. annua* suggest that genetic factors establishing individual plants as male or female may control sex expression by setting extreme endogenous levels of cytokinin and auxin (Louis 1989, Durand and Durand 1991).

Environmental factors

Environmental factors have been observed to influence sex expression in a number of plants species (reviewed by Freeman et al. 1980 and Korpelainen 1998). Naturally, environmental conditions are not stable over time; therefore the gender of a plant which is subject to varying conditions may change from season to season. Plants able to change their sexual state according to varying environmental conditions are sexually 'labile'. Even some dioecious species reported to have sex chromosomes have the potential of changing sex (*C. sativa*, see Freeman et al. 1980). Without much doubt, plants capable to change their sexual phenotype will have a selective advantage over individuals lacking this ability in patchy or changing environments. Both Freeman et al. (1980) and Korpelainen (1998) compiled an extensive list of species in which environmental factors have been demonstrated to influence plant sex expression. Generally, benign conditions (high CO₂, mild temperature, moist and nitrogen-rich soils, high light intensity) favour female sex expression, whereas poor conditions favour male sex expression. This strategy is in line with the observation that females bear a greater cost of reproduction than males due to additional costs of seed and fruit production.

In addition, several environmental factors are also known to affect the metabolism of plant hormones associated with sex expression (reviewed by Freeman et al. 1980 and Korpelainen 1998). For example, nitrogen availability is assumed to influence auxin metabolism. Also, photoperiod is known to affect the sexual phenotype: long days promote maleness and short days promote femaleness. Thereby

photoperiod directly influences the concentrations of endogenous regulatory growth substances which, in turn, reflect the environmental background. Again, those plants which express the appropriate phenotype in response to certain hormone levels reflecting either male- or female-favoured growth conditions consequently will have a selective advantage.

Genetic factors

In some species, such as sub-dioecious *Ecballium elaterium*, simple genetic mechanisms differentiate the sexes. Here, the allelic constitution at a single locus determines whether a plant shows a male, female or monoecious phenotype (Galán 1946, Mather 1949). Other species, such as dioecious *M. annua*, have several unlinked loci that in combination determine gender. In this species, three independently segregating genes that control sex expression have been identified (Louis 1989, Durand and Durand 1991). In several species, the sex determining genes are compiled into certain linkage groups and form sex chromosomes (reviewed by Matsunaga and Kawano 2001, and Charlesworth 2002). While most dioecious plant species have sex chromosomes that are morphologically indistinguishable (e.g. *Spinacia oleracea*, *A. officinalis*), few species possess morphological distinct sex chromosomes (e.g. *S. latifolia*, *C. sativa*) (Parker 1990). In the majority of plants investigated, males are the heterogametic sex with a male-specific and a female-specific sex chromosome (XY), and females are homogametic with two female-specific sex chromosomes (XX) (Westergaard 1958). In *S. latifolia*, the Y-chromosome is the largest among all other chromosomes (Donnison et al. 1996). The Y-chromosome of many male heterogametic animal species however, is small compared to the other chromosomes in the complement. Because they have no recombination partner in meiosis, Y-chromosomes potentially can accumulate deleterious mutations (Muller 1964). That is why these chromosomes are thought to express only a minimal number of genes. Apparently, sex chromosomes of dioecious plants have diverged from an autosomal pair more recently than animal sex chromosomes. A relatively novel theory states that dioecy and sex chromosomes originated in ancestral hermaphrodite species as a pair of homologous chromosomes (i.e. autosomes) (see e.g. Gorelick 2003). Due to differential methylation patterns – one chromosome had

more methyl groups attached (i.e. more genes become inactivated) to the sex-controlling region compared to the other – two distinct sexes were initially determined. Such epigenetic differences between the sex chromosomes could later become genetic, after mutations in inactivated genes occurred.

Among dioecious plant species with sex chromosomes, two types of mechanisms to determine sex expression have been described (e.g. reviewed by Ainsworth 1998): (1) the active Y chromosome system (e.g. *S. latifolia*, *A. officinalis*), which resembles the sex determination scheme in mammals, and (2) the X-to-autosome ratio mechanism (e.g. *H. lupulus*, *Rumex acetosa*), which has similarity to the mechanism operating in *Drosophila*. If sex expression is controlled by a Y-chromosome active system, the Y-chromosome is decisive in determining gender. In *S. latifolia* (females are XX and males are XY), three different regions on the Y-chromosome have been identified as having separate functions in sex determination. One locus contains a genetic factor that suppresses female organ formation, a second one contains a male fertility factor, and a third locus includes a gene (or genes) for male organ formation (Donnison et al. 1996). With the X-to-autosome ratio mechanism on the other hand, gender is determined by the ratio between X-chromosomes and autosome sets (an autosome is a chromosome that is not a sex chromosome). In diploid *R. acetosa* (females are XX and males are XY_1Y_2), plants are female if the X-to-autosome ratio is 1.0 (XX:AA) or more than 1.0, whereas individuals are male if the ratio is 0.5 (XYY:AA) (Ainsworth 1998). A ratio between 0.5 and 1.0 leads to a hermaphrodite phenotype (e.g. in triploid *R. acetosa* with XXYY:AAA). Although the Y-chromosome does not carry the major genes for sex differentiation, the presence of two Y-chromosomes is essential to complete meiosis.

Apart from the conventional systems described above, polygenic sex determination, in which sex is determined by the sum of genetic effects of genes (each with a minor effect) distributed throughout the chromosome complement (Bull et al. 1982, Bulmer and Bull 1982), is a subject that recently received increasing attention when discussing sex determination mechanisms in animals and plants (e.g. Premoli et al. 1995, Ehlers et al. 2005). This may be because our view on sexuality has changed over time and we are now aware more than ever that sex determination, which was often regarded as a sim-

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ple case, is really rather complex. With polygenic sex determination, sex is a quantitative character (threshold character) in which the discrete sexual phenotype of male and female individuals is determined by an underlying, continuous character X . So individuals born with X larger than the threshold T belong to one sex or to the other sex if $X < T$.

EVOLUTION OF DIOECY

Since the 19th century and the publication of Darwin's classical work *The Different Forms of Flowers on Plants of the Same Species* (1877), there has been increasing interest in understanding (1) from which types of breeding systems did dioecy originate, and (2) which types of selective forces resulted in the evolution of dioecy? Here, I will focus on the latter question. Various authors (e.g. Baker 1959, Carlquist 1974) have argued that dioecy has evolved as a mechanism to reduce inbreeding (outbreeding hypothesis). Spatial separation of male and female organs in the flower (herkogamy), temporal displacement in the maturation of male and female organs (dichogamy), and separate male and female flowers (monoecy) are alternative plant strategies that promote outbreeding. However, while the above mentioned mechanisms prevent self-pollination within a flower, self-pollination still may occur on the same plant (geitonogamy) and therefore may result in offspring with inferior quality. In contrast, self-incompatibility is an effective outbreeding device. So in those cases where close relatives that are not dioecious are self-incompatible, we do not expect a species to have evolved dioecy. A study by Delesalle and Muenchow (1992) compared two sister species of *Sagittaria* belonging to different sex systems. *S. latifolia* is sub-dioecious and has both purely monoecious populations and predominantly dioecious populations. *S. australis*, on the other hand is strictly monoecious. Seed set, germination rate, seedling mortality and size of six week old seedlings were measured in selfed and outcrossed progeny. For outcrossing to be favoured in natural populations, outcrossed progeny must be twice as fit as selfed progeny. The authors demonstrated that outcrossed progeny indeed performed almost twice as better compared to selfed progeny. Possibly, if Delesalle and Muenchow (1992) would have included fitness measurements of progeny older than six weeks, inbreeding depression of selfed progeny may have increased in

later life stages. Furthermore, all fitness measurements were carried out under laboratory conditions. Field conditions which are generally more stressful may have resulted in larger differences in performance between selfed and outcrossed progeny. Follow-up studies on the genus *Sagittaria* convincingly showed that inbreeding depression was high enough to corroborate the outbreeding hypothesis for the evolution of dioecy (Dorken et al. 2002, Barrett 2003).

Other authors (e.g. Bawa 1980, Givnish 1980), in turn, favoured an ecological hypothesis for the evolution of dioecy. For example, Givnish (1980) emphasized that seed dispersers (e.g. birds) preferentially visit plants producing high numbers of fruits compared to plants that do not. In order to evolve unisexuality, an increase in female reproductive effort must result in a disproportional increase in female fitness. A review of available data by de Jong and Klinkhamer (2005) suggested that this hypothesis could not be rejected and thus may complement the ecological hypothesis. Yet, the idea of Givnish (1980) does not hold for dioecious species, in which seeds are dispersed by abiotic factors. For example in wind-dispersed plant species, an increase in the total number of seeds would lead to a decrease in the fraction of seeds that is dispersed to suitable sites because in most wind-dispersed species the seeds fall near the parental plant (e.g. *U. dioica*, McKey 1975).

URTICA DIOICA AS A STUDY ORGANISM

Originally *Urtica dioica* L. (stinging nettle) was a frequent study object of population ecological studies (reviewed by Šrutek and Teckelmann 1998). There is a large body of literature available dealing with some aspect of autecology and/or population ecology of *U. dioica*. For our study, there are several reasons to investigate the problem of sex ratio variation and sex determination in this plant species. First of all, there is considerable between-family variation in progeny sex ratio of *U. dioica* (de Jong et al. 2005), making it an interesting study object to unravel the mechanism behind this variation. Secondly, *U. dioica* flowers within two months from germination under laboratory conditions and due to its capability of vegetative growth, genotypes of interest can be cloned and kept in tissue culture. Thirdly, the existence of bisexual plants beside male and female individuals in natural populations of *U. dioica* allows us to perform

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crosses aimed at identifying the heterogametic sex (Westergaard 1958). Also, further studies can stand to benefit from the occurrence of such bisexual individuals while exploring the evolution of dioecy in this particular plant species.

THESIS OUTLINE

This thesis will first document on variation in progeny sex ratios among individual female plants at our field site in Meijendel (*Chapter 2*). Next, we show that there is also considerable sex ratio variation among male and female flowering shoots in 26 natural populations studied (*Chapter 3*). Additionally, we studied life history traits of male and female clones to detect sex-specific differences that might have contributed to the sex ratio bias observed in the field. Our results indicate that the sex ratio bias in natural populations may be a consequence of a bias that already originated in the primary sex ratio. Next, we investigated physiological, environmental (*Chapter 4*) and genetic (*Chapter 5*) aspects of sex determination. For the latter aspect, a series of crosses including male, female and monoecious plants of *U. dioica* was performed. These experiments were designed to investigate whether sex ratio variation in the primary sex ratio is due to environmental sex determination (ESD) or genetic sex determination (GSD) solely or due to an interaction of both. Genetic mapping of sex-linked markers was used as a supplementary tool to analyze the genetic mechanism of sex determination (*Chapter 6*). Finally, to study the inheritance pattern of the sex ratio trait, crosses were performed between individual male and female plants from different sex ratio families (*Chapter 7*).